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Document Title

TESTING OF THE CELL TRANSFORMATION ACTIVITY OF HE 1002 (FINAL REPORT) WITH COVER LETTER DATED 112291

Chemical Category

4,4-DIPHENYLMETHANE DIISOCYANATE (101-68-8)

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Mobay Corporation
Health, Environment, Safety
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Mobay Road
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November 22, 1991

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The information required at 40 CFR 716.30 is given below.

Chemical Name:	4,4-Diphenylmethane diisocyanate
CAS No:	101-68-8
Name of Study:	Testing of the Cell Transformation Activity of HE 1002
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If you have any questions, please contact me.

Sincerely,

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Attachments
Certified Mail No.: P 276 377 332

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TESTING OF THE CELL TRANSFORMATION ACTIVITY OF HE 1002

IRI Project No. 703879

Bayer Study No. HE 1002/012

IRI Inveresk Research International

K 2097

INVERESK RESEARCH INTERNATIONAL
Report No. 1862

CONFIDENTIAL

TESTING OF THE CELL TRANSFORMATION ACTIVITY OF HE 1002

IRI Project No. 703879

Bayer Study No. HE 1002/012

Authors:

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November 1980

AUTHENTICATION

"I, the undersigned, hereby declare that this work was performed under my supervision, according to the procedures herein described and that this report represents a true and accurate record of the results obtained."

W.J. Harris, B.Sc., Ph.D.
Principal Investigator

Project No. 703879

Report No. 1862

CONTENTS

QUALITY ASSURANCE AUTHENTICATION

The execution of this type of short-term study is not individually inspected. The processes involved are inspected at intervals according to a pre-determined schedule.

The report has been audited by IRI Quality Assurance personnel according to the appropriate Standard Operating Procedure and is considered to describe the methods and procedures used in the study. The reported results accurately reflect the original data of the study.

IRI PROJECT NO. 703879 Report No. 1862

Signed Andrew Waddell Date 31st August 1981

Quality Assurance Manager

STAFF INVOLVEMENT

Principal Investigator:	W.J. Harris, B.Sc., Ph.D.
Project Leader:	A. Poole, B.Sc., Ph.D.
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SUMMARY

The compound HE 1002 was tested for potential carcinogenicity in a cell culture transformation assay.

Cells treated with HE 1002 in the presence of S-9 caused at the LC_{50} 10.1, 4.2 and 5.5 fold increases in transformation frequency as compared to negative control. In the absence of S-9 1.1 and 9 fold increases were observed.

These significant increases in the transformation frequencies at the LC_{50} were also accompanied by increases in absolute numbers of transformed colonies in cells treated with HE 1002.

Thus, by the criterion used in this laboratory HE 1002 is considered to be a potential cell transforming agent.

INTRODUCTION

HE 1002 was tested for potential carcinogenicity by a cell culture transformation assay.

The assay is based on the observations that, following exposure to a carcinogen, malignantly transformed cells unlike their non-malignant counterparts, will undergo sufficient divisions in a soft agar medium to produce macroscopically visible colonies (Shin, et al 1975 and di Mayorca et al 1973).

As normal laboratory stocks of BHK 21 C13 cells contain spontaneously transformed variants which frequently cause a high background of macroscopic colonies when plated in soft agar, i.e. $50/10^6$ plated cells, a subline selected from normal stocks able to express the transformed phenotype in response to treatment with chemical carcinogens, but with a low spontaneous transformation rate, was used in these experiments. The procedure used otherwise is a modification of that described by Styles (1977).

This work was carried out at the Inveresk Gate Laboratories of Inveresk Research International, Musselburgh, EH21 7UB between August and October 1980.

MATERIALS AND METHODS

Sterile procedures were used throughout preparation of materials and experimental methods.

Chemicals

The cream solid labelled HE 1002 was received from Bayer AG, Wuppertal, West Germany on the 15 August 1980. The sample was stored in the dark at room temperature.

The positive control substances N-methyl-N'-nitro-N-nitroso-guanidine (MNNG) and 2-acetamidofluorene (2-AAF) were obtained from Sigma London Chemical Company Limited, U.K. and Koch-Light Laboratories, Colnbrook, Bucks., respectively.

The polychlorinated biphenyl mixture, Aroclor 1254, was received from Analabs Incorporated, Newhaven, Connecticut, U.S.A.

Cells

These were a subline from a sample of EHK 21 C13 of Syrian hamster kidney cells provided by the Imperial Cancer Research Fund.

Culture Media

For cell cultivation, the medium was Dulbecco's Modification of Eagle's Minimum Essential Medium (DMEM), with a concentration of sodium bicarbonate of 2 g/l to permit equilibration with a gas phase of 5% CO_2 in air, it was supplemented with newborn calf serum (10%, v/v), selected by pre-screening a number of serum samples for cloning efficiency, and contained gentamicin (50 µg/ml).

For incubation with the compound under test, the cells, in order to prevent clumping during exposure, were suspended in Eagle's Minimum Essential Medium modified for suspension cultures (MEMS), buffered with 20 mM HEPES, the S-9 mixture was added to ascertain if the carcinogenic potential of the compound was affected by the metabolic activity of liver enzymes.

Culture media and sera were obtained from Flow Laboratories, Irvine, Scotland and other chemicals from Sigma London Chemical Company Limited.

Maintenance of Stock Cultures

These were grown as monolayers in Nunc flasks and, in order to minimise selection for spontaneously transformed variants which accumulate when stocks reach confluence (Kao et al 1975), the cells were never grown to confluency.

For subcultivation, the medium was removed and the cells treated for one minute in a solution of 0.25% trypsin in phosphate buffered balanced salt solution containing EDTA (0.002% w/v). After removal of the enzyme solution, the flasks were left to incubate at 37°C until the cells began to detach from the plastic. 5 ml of fresh culture medium was then added and the cells brought into suspension by repeated aspiration through a sterile 10 ml pipette. Aliquots of the cell suspension were then added to medium in fresh culture flasks, the usual ratio for division of monolayers being 1:30.

Preparation of S-9 Mix

Animal Treatment

Male rats of the Bantin and Kingman Fischer strain weighing between 200 and 300 g were injected (i.p.) with Aroclor 1254

dil'ed in corn oil to a concentration of 200 mg/ml at a dosage of 500 mg/kg body weight to induce microsomal enzyme activity.

The animals were killed by cervical dislocation 5 days after treatment following a 16 h fasting period.

Preparation of the 9,000 g Supernatant Fluid from Livers

Und[illegible] aseptic conditions, livers from the freshly killed animals were carefully removed and weighed in sterile beakers containing ice-cold 0.15 M-KCl. Further ice-cold salt solution was added to the beakers to give a final volume equivalent to 3 times the weight of the livers which were subsequently finely chopped with sterile long-handled scissors before being transferred to a Potter homogeniser.

The chopped livers were homogenised in a sterile glass vessel by 8 strokes of a Teflon pestle rotating at about 1,200 r.p.m. The homogenate was then transferred to sterile polypropylene tubes and centrifuged at 9,000 g for 10 min at 0°C.

The supernatant fraction was decanted into sterile containers and stored in liquid nitrogen until required.

S-9

Under aseptic conditions, the S-9 mix was prepared as follows:

Ice-cold 0.05 M-phosphate buffer, pH 7.4, was added to pre-weighed co-factors to give a final concentration in the S-9 mix of:

NADP-di-Na-salt	4 mM (= 3.366 mg/ml)
Glucose-6-phosphate-di-Na-salt	5 mM (= 1.521 mg/ml)
$MgCl_2.6H_2O$	8 mM (= 1.626 mg/ml)
KCl	33 mM (= 2.460 mg/ml)

The solution was sterilised by passage through a 0.22 µm filter and mixed with the liver 9,000 g supernatant fluid in the following proportions:

co-factor	1 part
liver preparation	1 part

Preliminary Toxicity Test

This was done to establish the range of concentrations to be used in the cell transformation assay.

The cells were harvested and suspended in growth medium as for subculture, sedimented by centrifugation at 200 g for 5 min and resuspended in MEMS at a density of 10^6 cells/ml. 1 ml samples were then pipetted into plastic universal bottles (Sterilin Limited).

The test material was dissolved in dimethylsulphoxide at concentrations of 100, 10, 1.0, 0.1 and 0.01 mg/ml and 10 µl samples were added to duplicate cell suspensions to give final concentrations of 1,000, 100, 10, 1.0 and 0.1 µg/ml.

After incubation for 4 h at 37°C in an orbital shaker at 150 r.p.m. (New Brunswick, New Jersey) the bottles were centrifuged at 100 g for 4 min and the supernatant medium in each was replaced with 10 ml of growth medium. Each cell suspension was mixed thoroughly in a variable speed vibratory shaker (Fisons Limited), and a 35 µl sample was dispersed evenly in 5 ml growth medium in a 50 mm tissue culture dish (Nunclon Delta). The dishes were incubated for 7 days at 37°C in a humid atmosphere of 5% CO_2 in air. The cultures were then fixed with methanol and stained with Giemsa and the colonies in each dish were manually counted.

From the toxicity data 5 doses were selected for use in the transformation assays.

Cell Transformation Assay

Triplicate plates were used at each dose level of test compound and all experiments were repeated.

For all tests the procedure was described below.

Samples of BHK 21 C13 cells were harvested, suspended in MEMS with or without S-9 mix (5% v/v), and distributed among sterile plastic universal bottles as in the preliminary toxicity test.

The compound was dissolved in dimethylsulphoxide at the following concentrations:

200, 100, 50, 25 and 12.5 mg/ml

Triplicate cell suspensions, with and without S-9 mix, received 10 µl samples of the test solutions.

The positive control compounds were N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) for the S-9-free suspensions and 2-acetamidofluorene (2-AAF) for the S-9-supplemented suspensions. These were dissolved in dimethylsulphoxide in concentrations giving on dilution 1-100 in cell suspension the following levels:

MNNG: 0.3, 0.15, 0.075 and 0.375 µg/ml

2-AAF: 600, 300, 150 and 75 µg/ml

Triplicate negative control suspensions received 10 µl of dimethylsulphoxide.

Incubation of the suspensions, resuspension of the cells in growth medium and preparation of the cultures for estimation of cell survival were carried out as in the preliminary toxicity test.

After removal of the 35 µl samples from each bottle for the measurement of toxicity, 625 µl of 5% solution of agar (Difco Noble) in distilled water was mixed rapidly with the remaining suspension and the mixture poured into a 50 mm bacteriological Petri dish which was left at 4°C for 5 min to allow the agar to gel. The dishes were then incubated for 21 days at 37°C in sealed containers in a humid atmosphere of 5% CO_2 in air.

Quantitation of Transformation

After a 7 day incubation period, the colonies growing in the tissue culture dishes were fixed for 30 min in methanol and stained with Giemsa. The colonies in each dish were counted manually and cell viability at each concentration of test compound was expressed as a percentage of the survivors in the negative control cultures, i.e. those dosed with dimethyl-sulphoxide only.

After 21 days incubation, the cells growing in soft agar were examined with a New Brunswick Biotran II Automatic Colony Counter (New Brunswick, New Jersey) and the number of transformants regarded as colonies with a diameter of > 0.22 mm (Bouck et al 1976), were counted. From the results of the transformation and survival assays the transformation frequency (number of transformed colonies/10^5 surviving cells) was calculated for each dose.

Evaluation

In the cell transformation test described by Styles (1977), a transformation frequency, at LC_{50} of the compound under test, of 5 times that for the spontaneous transformation frequency is considered to indicate potential carcinogenicity. Using this method in tests on 120 compounds 91% of carcinogens and 97% of non-carcinogens were correctly distinguished, Purchase et al (1976). This is one criterion applied in the present study to indicate potential carcinogenicity of the test agent. In addition, if the test compound is relatively non-toxic or is insufficiently soluble to achieve a concentration resulting in 50% toxicity, a 2-fold increase in the absolute number of transformed colonies per dish at 2 doses is considered to indicate a positive response.

The clone of BHK 21 C13 cells used in these studies may in particular experiments give very low spontaneous transformation levels, i.e. 1 or 2. If this occurs the values obtained with the treated cells are related instead to the average spontaneous transformation rate (5 transformed colonies) found from a series of tests.

RESULTS AND DISCUSSION

From the results of the initial toxicity test (Table 1), a top dose of 2000 µg/ml was selected for the transformation assays. The addition of S-9 mix to the incubation mixture appeared to have little effect on the toxicity of HE 1002, the mean LC_{50} values in the presence and absence of S-9 mix being 1,320 (Figures 1-3) and 1290 µg/ml (Figures 4 and 5) respectively.

In the presence of S-9 mix HE 1002, at the LC_{50}, caused 10.1, 4.2 and 5.5 fold increases in transformation frequencies relative to that of the negative controls (Figures 1-3). These significant increases in the transformation frequencies at the LC_{50} were also accompanied by large increases in the absolute numbers of transformed colonies in cells treated with relatively non-toxic concentrations of HE 1002 in 2 out of the 3 experiments (Tables 3 and 4).

In the absence of S-9 mix, 1.1 and 9.4 fold increases in transformation frequencies at the LC_{50} were measured (Figures 4 and 5).

These together with absolute increases in the numbers of transformed colonies in cells treated with HE 1002 below the LC_{50} level also suggests a positive response (Tables 5 and 6).

The results obtained in this study indicate that HE 1002 should be regarded as a potential cell transforming agent.

CONCLUSION

By the criterion used in this assay, HE 1002 showed evidence of cell transforming potential.

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(3) Kao, F. and Puck, T.T. (1975), Genetics, 79, 343.

(4) Purchase, I.F.H., Longstaff, E., Ashbey, J., Styles, J.A., Anderson, D., Lefeure, P.A. and Westwood, F.R. (1976), Nature, 264, 624.

(5) Shin, S., Freedman, V.H., Risser, R. and Pollack, R. (1975), Proc. Nat Acad. Sci., 72, 4435.

(6) Styles, J. (1977), Brit. J. Cancer, 36, 558.

TABLE 1

Cytotoxicity Test

Project No: 703879
Contractor: Bayer
Operator(s): Alan Poole
Date of test: 19 August 1980

Substance: HE 1002
Activation: None
Liver preparation date: None
Cell culture batch: 79.6 (4.1)
Date survival assay counted: 26 August 1980

Substance Quantity µg/ml	Survival Assay Colonies/plate		Survival as Percentage of Negative Control
		Average	
1000	82, 67	74.5	45
100	141, 166	153.5	92
10	153, 156	154.5	93
1.0	152, 171	161.5	97
0.1	171, 177	174	104
DMSO Control	170, 164	167	100

TABLE 2

Cell Transformation

Project No: 703879
Contractor: Bayer
Operator(s): Alan Poole, Nick Hunter, Catherine Green
Date of test: 28 August 1980
Date survival assay counted: 3 September 1980
Date transformation assay counted: 17 September 1980

Substance: HE 1002
Activation: Aroclor-induced Fischer Rat
Liver preparation date: 13 August 1980
Cell culture batch: 78.13 (2)

Substance Quantity µg/ml	Survival Assay Colonies/plate	Average	Transformation Assay Colonies/plate	Average	Viable Count x 10^4	Transformed Colonies/10^5 Viable Cells	Survival as Percentage of Negative Control
HE 1002							
2000	1, 0, 0	0.3	0, •, 0	0	0.0086	0	0
1000	6,128, 158	97.3	20, 40, 17	25.7	2.78	92	51
500	130,149, 142	140.3	57, 71, 71	66.3	4.01	165	74
250	145,165, 183	164.3	0, 77, 29	35.3	4.70	75	87
125	177,160, 212	183	53, 36, 91	60	5.23	115	97
2-AAF							
600	0, 0, 1	0.3	0, 0, 3	1	0.0086	1163	0
300	13, 1, 1	5	0, 6, 6	4	0.14	286	3
150	8, 22, 37	22.3	2, 7, 8	5.7	0.64	89	12
75	216,170, 177	187.7	•, 3, 8	5.5	5.36	10	99
DMSO Control	184,186, 198	189.3	5, 7, 2	4.7	5.41	9	100

• Petri dish contaminated

TABLE 3

Cell Transformation

Project No: 703879
Contractor: Bayer
Operator(s): Alan Poole, Nick Hunter, Catherine Green
Date of test: 8 September 1980
Date survival assay counted: 19 September 1980
Date transformation assay counted: 29 September

Substance: HE 1002
Activation: Aroclor-induced Fischer Rat
Liver preparation date: 13 August 1980
Cell culture batch: 78.13 (4)

Substance Quantity µg/ml	Survival Assay Colonies/plate		Transformation Assay Colonies/plate		Viable Count x 10^4	Transformed Colonies/10^5 Viable Cells	Survival as Percentage of Negative Control
		Average		Average			
HE 1002							
2000	0, 0, 0	0	0, 0, 0	0	0	0	0
1000	369,185, 170	241.3	25,12, ●	18.5	6.90	27	62
500	280,263, 291	278	25,41, 35	33.7	7.94	42	72
250	313,420, 383	372	6,33, ●	19.5	10.63	18	96
125	319,412, 383	371.3	42,13, ●	27.5	10.61	26	96
2-AAF							
600	2, 8, 2	4	1, 4, 4	3	0.11	273	1
300	3, 7, 1	3.7	4, 2, ●	3	0.11	273	1
150	8, 7, 9	8	8,10, 9	9	0.23	391	2
75	451,409, 272	377.3	12, 7, ●	9.5	10.78	9	98
DMSO							
Control	484,354 321	386.3	5, 5, ●	5	11.04	5	100

● Petri dish contaminated

TABLE 4

Cell Transformation

Project No: 703879
Contractor: Bayer
Operator(s): Alan Poole, Nick Hunter, Catherine Green
Date of test: 11 September 1980
Date survival assay counted: 22 September 1980
Date transformation assay counted: 2 October 1980

Substance: HE 1002
Activation: Aroclor-induced Fischer Rat
Liver preparation date: 13 August 1980
Cell culture batch: 79.5 (12)

Substance Quantity µg/ml	Survival Assay Colonies/plate		Transformation Assay Colonies/plate		Viable Count x 10^4	Transformed Colonies/10^5 Viable Cells	Survival as Percentage of Negative Control
		Average		Average			
HE 1002							
2000	0,156, 282	146	33,11, ●	22.0	4.17	53	44
1000	280,197, 240	239	4, 3, ●	3.5	6.83	5	73
500	288,251, 261	266.7	24,34, ●	29.0	7.62	38	81
250	304,273, 258	278.3	47,29, 26	34.0	7.95	43	85
125	274, ●, 290	282.0	0, 9, 11	6.7	8.06	8	86
2-AAF							
600	48, 44, 52	48.0	7, 4, 2	4.3	1.37	31	15
300	104, 50, 131	95.0	●,12, 9	10.5	2.71	39	29
150	163,107, 129	133.0	41,40, 34	38.3	3.80	101	40
75	241,255, 256	250.7	0, 4, 0	1.3	7.16	2	76
DMSO							
Control	353,332, 301	328.7	4, ●, 11	7.5	9.39	8	100

● Petri dish contaminated

TABLE 5

Cell Transformation

Project No: 703879
Contractor: Bayer
Operator(s): Alan Poole, Nick Hunter, Catherine Green
Date of test: 8 September 1980
Date survival assay counted: 22 September 1980
Date transformation assay counted: 29 September 1980

Substance: HE 1002
Activation: None
Liver preparation date: None
Cell culture batch: 78.13 (4)

Substance Quantity µg/ml	Survival Assay Colonies/plate		Transformation Assay Colonies/plate		Viable Count x 10^4	Transformed Colonies/10^5 Viable Cells	Survival as Percentage of Negative Control
		Average		Average			
HE 1002							
2000	1, 2, 0	1	0, 0, 0	0	0	0	0
1000	188,171, 218	192.3	8,18, •	13	5.49	24	73
500	243,188, 221	217.3	28,23, •	25.5	6.21	41	83
250	203,196, 213	204	8,26, 10	14.7	5.83	25	78
125	240,256, 258	251.3	7, 6, •	6.5	7.18	9	96
MNNG							
0.3	0, 0, 0	0	0, 0, 0	0	0	0	0
0.15	65, 71, 0	45.3	18,17, 8	14.3	1.29	111	17
0.075	212,165, 224	200.3	14,10, 13	12.3	5.72	22	77
0.0375	242,227 270	246.3	6, 4, 1	3.7	7.04	5	94
DMSO							
Control	316,236, 233	261.7	6,11, 15	10.7	7.48	14	100

• Petri dish contaminated

TABLE 6

Cell Transformation

Project No: 703879
Contractor: Bayer
Operator(s): Alan Poole, Nick Hunter, Catherine Green
Date of test: 11 September 1980
Date survival assay counted: 22 September 1980
Date transformation assay counted: 2 October 1980

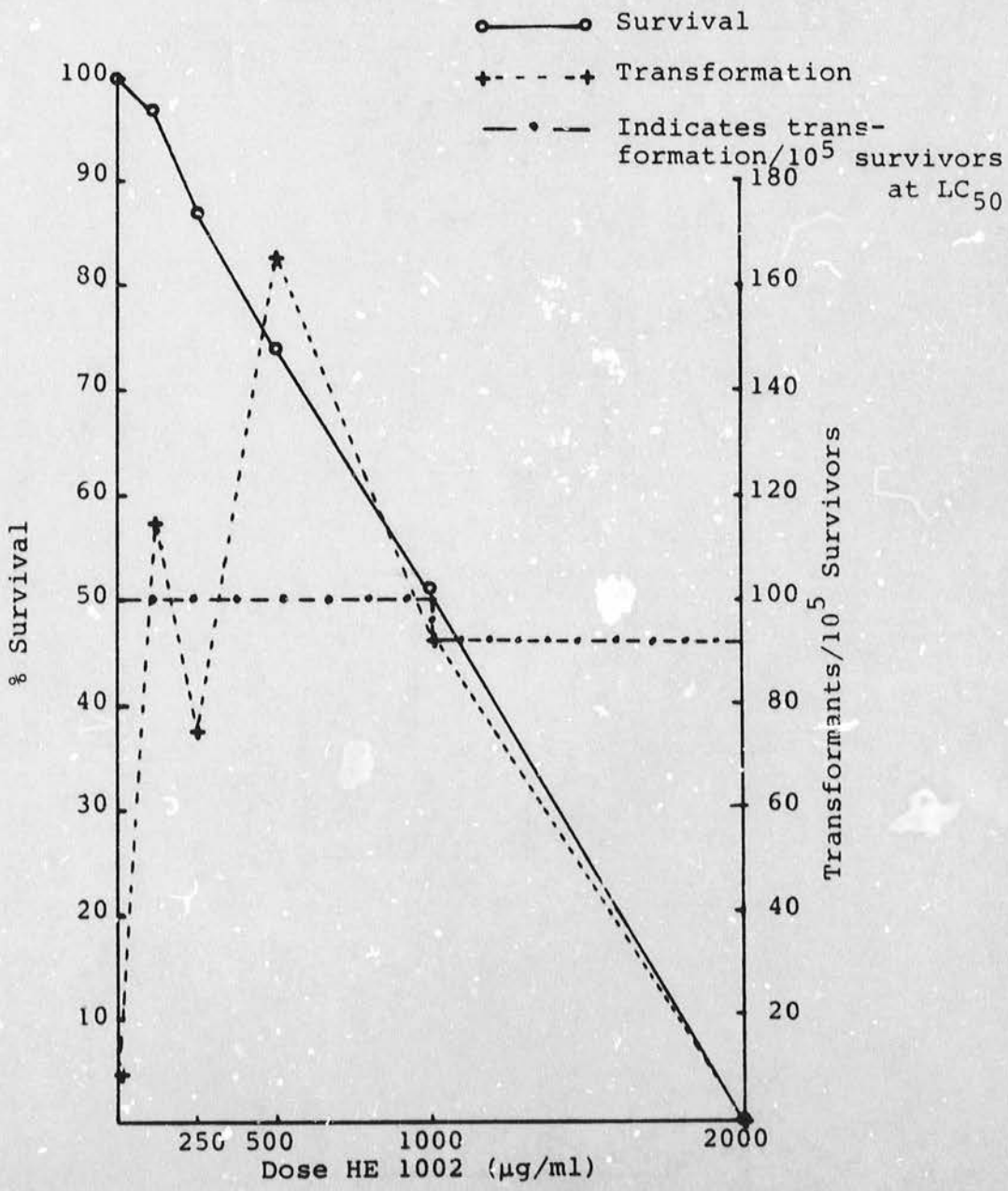
Substance: HE 1002
Activation: None
Liver preparation date: None
Cell culture batch: 79.5 (12)

Substance Quantity µg/ml	Survival Assay Colonies/plate		Transformation Assay Colonies/plate		Viable Count x 10^4	Transformed Colonies/10^5 Viable Cells	Survival as Percentage of Negative Control
		Average		Average			
HE 1002							
2000	0, 0, 0	0	0, 0, 0	0	0	0	0
1000	217,122, 197	178.7	28,28, 27	27.7	5.11	54	58
500	215,154, 195	188	81,15, 19	38.3	5.37	71	61
250	218,220, 216	218	33,36, 16	28.3	6.23	45	71
125	247,264, 267	259.3	35,12, •	23.5	7.41	32	85
MNNG							
0.3	35, 39, 63	45.7	16,26, 24	22.0	1.31	168	15
0.15	246,141, 233	206.7	11,16, 11	12.7	5.91	21	67
0.075	291,222, 246	253	3,17, 8	9.3	7.23	13	83
0.0375	313,282, 261	285.3	•,11, 5	8.0	8.15	10	93
DMSO							
Control	354,291, 275	306.7	6, 4, 4	4.7	8.76	5	100

• Petri dish contaminated

FIGURE 1

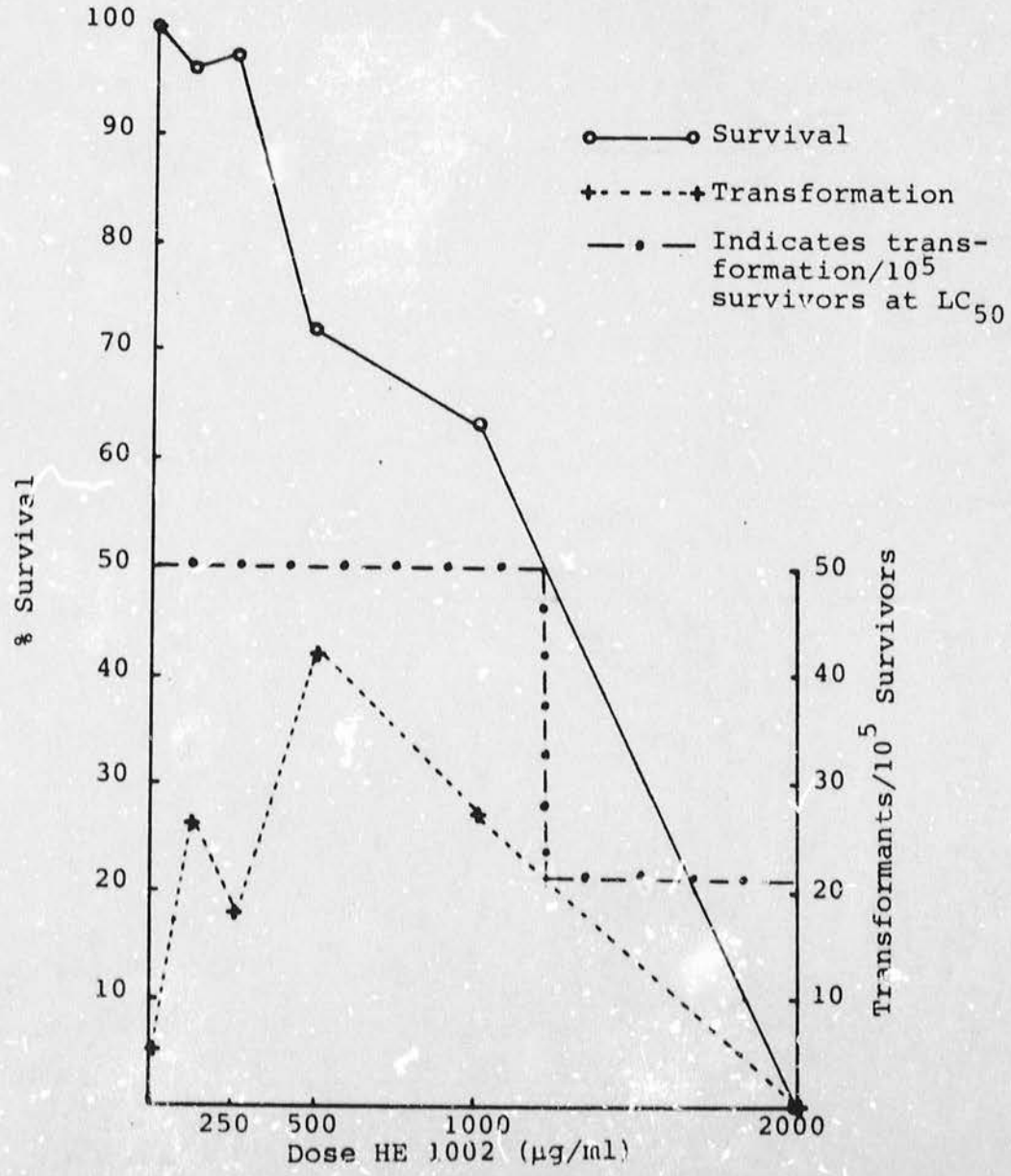
Survival and Transformation Frequency of BHK 21 C13 Cells Treated with HE 1002 in the Presence of S-9 Activation
Graph of Results from Table 2



Transformation frequency of HE 1002 at LC_{50} = 91
Transformation frequency of 2-AAF at LC_{50} = 50
Transformation frequency of DMSO = 9

FIGURE 2

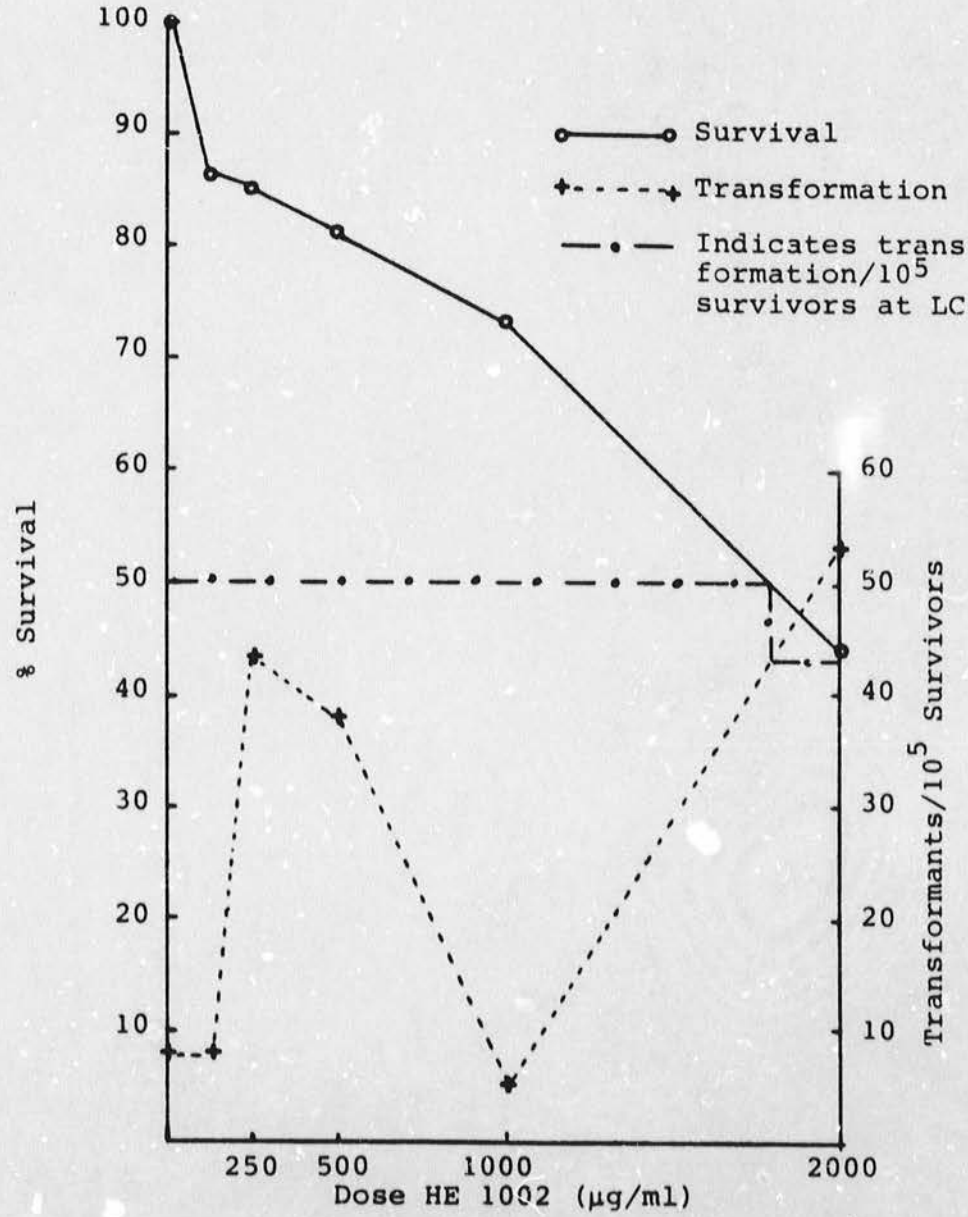
Survival and Transformation Frequency of BHK 21 C13 Cells Treated with HE 1002 in the Presence of S-9 Activation
Graph of Results from Table 3



Transformation frequency of HE 1002 at LC_{50} = 21
Transformation frequency of 2-AAF at LC_{50} = 200
Transformation frequency of DMSO = 5

FIGURE 3

Survival and Transformation Frequency of BHK 21 C13 Cells Treated with HE 1002 in the Presence of S-9 Activation
Graph of Results from Table 4

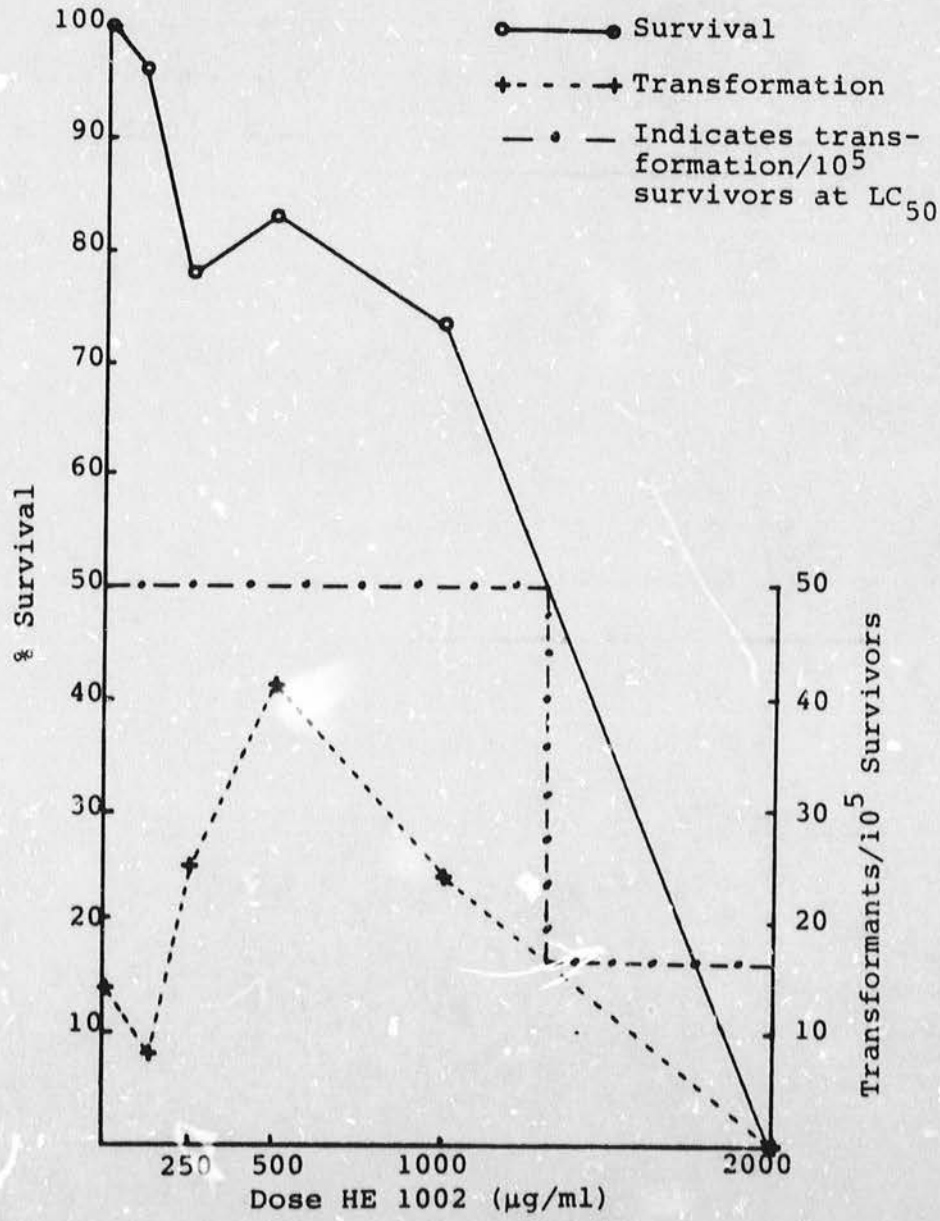


Transformation frequency of HE 1002 at LC_{50} = 44
Transformation frequency of 2-AAF at LC_{50} = 74
Transformation frequency of DMSO = 8

FIGURE 4

Survival and Transformation Frequency of BHK 21 C13 Cells Treated with HE 1002 in the Absence of S-9 Activation

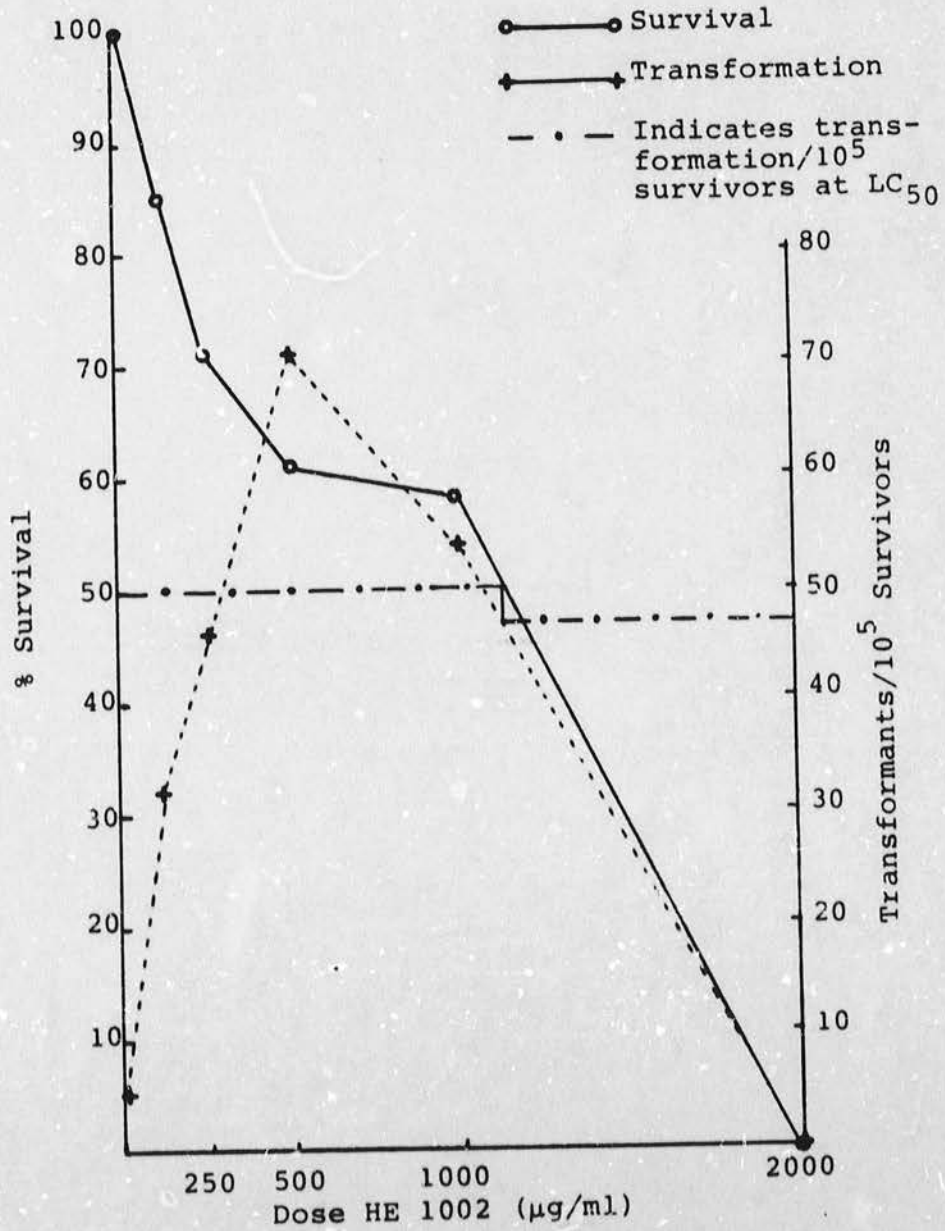
Graph of Results from Table 5



Transformation frequency of HE 1002 at LC_{50} = 16
Transformation frequency of MNNG at LC_{50} = 62
Transformation frequency of DMSO = 14

FIGURE 5

Survival and Transformation Frequency of BHK 21 C13 Cells Treated with HE 1002 in the Absence of S-9 Activation
Graph of Results from Table 6



Transformation frequency of HE 1002 at LC_{50} = 47
Transformation frequency of MNNG at LC_{50} = 69
Transformation frequency of DMSO = 5

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